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Detection of T-Cell Receptor Delta Gene Rearrangement in T-Cell Malignancies by Clonal Specific Polymerase Chain Reaction and Its Application to Detect Minimal Residual Disease

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A clonal-specific polymerase chain reaction technique to detect T-cell receptor delta gene rearrangement in acute lymphoblastic leukaemia (ALL) and non-Hodgkin's lymphoma (NHL) was evaluated. It was applied to detect minimal residual disease. A sensitive and specific technique to detect minimal residual disease for T-cell malignancies was explored. Southern analysis and polymerase chain reaction (PCR) were used to detect the rearranged V-D-J segment of T-cell receptor delta (TCRδ) gene from malignant cell specimens of patients with leukemia and lymphoma of T-cell lineage. The PCR product was sequenced and from the DNA sequences of the V-D-J region, a 3′ anti-sense primer was designed and synthesized for clonal specific PCR (CS-PCR). T-cell receptor delta (TCR-δ) gene rearrangement was studied in 40 cases of acute leukaemia and lymphoma of T-cell lineage at diagnosis. Using Southern analysis, the positive rates were 28 and 32% for the 18 T-lymphoma and 22 T-ALL, respectively. A one stage Polymerase Chain Reaction (PCR) technique was used to detect the rearrangement in Southern positive cases and the PCR positive rates were 80 and 86%, respectively. The PCR technique had a sensitivity of 0.1%. Serial follow-up marrow specimens were available from 4 T-ALL patients following chemotherapy for monitoring of minimal residual disease. Their PCR products were DNA sequenced. A 3′ primer was designed for each case for a clonal specific (CS) PCR. The technique had a sensitivity of 0.003%. It was applied to detect minimal residual disease in serial follow-up marrow samples. The first patient had persistent negative CS-PCR results and enjoyed continuous remission for more than 3 years. The second patient with negative one stage PCR but positive CS-PCR results had eventual relapse of leukaemia. The other two patients never achieved a morphological remission. These preliminary results appeared to support the usefulness of these PCR techniques in detecting minimal residual disease and predicting relapses for ALL. However, further clinical correlation in larger populations of patients is necessary. © 1996 Wiley-Liss, Inc.

Key words: acute lymphoblastic leukemia, non-Hodgkin's lymphoma, T-cell receptor delta, polymerase chain reaction

INTRODUCTION

With modern chemotherapy, a complete remission (CR) can be achieved in most patients with acute lymphoblastic leukemia. CR is conventionally defined as the presence of less than 5% blast cells in the remission marrow under light microscopy. Although some of these CR patients may have prolonged remission and are possibly cured, the disease still relapses in many of them. This is probably due to the presence of residual leukemic cells in the marrow. The development of highly sensitive methods in detecting minimal residual disease (MRD) may help to identify patients who may require more aggressive therapy early, such as marrow transplantation, as well as those who are likely to remain in remission.

Immunoglobulin and T-cell receptor gene rearrangement studies have been successfully applied to

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TABLE I. The Panel of Monoclonal Antibodies

Monoclonal	CD	Specificity	Sources
B cell markers			
B4	CD19	Pan B cell	Dako (Carpinteria, CA)
B1	CD20	Pan B cell	Coulter (Hialeah, FL)
Pan B	CD22	Pan B cell	Dako
J5	CD10	Common ALL antigen	Coulter
Anti-lambda	—	Lambda light chain	Dako
Anti-kappa	—	Kappa light chain	Dako
T cell markers			
T6	CD1	Mature thymocytes	Coulter
T11	CD2	E rosette receptor antigen	Coulter
Leu 4	CD3	Mature T cell	Becton-Dickinson (San Jose, CA)
T1	CD5	Pan T cell	Dako
T2	CD7	T and B subsets	Dako
T4	CD4	T helper cell	Coulter
T8	CD8	T suppressor cell	Coulter
Activation and natural killer cells markers			
I2	—	HLA-DR related antigen	Coulter
IL2R	CD25	Interleukin-2 receptor	Coulter
Ki-1	CD30	Leucocyte activation antigen Reed-Sternberg cell	Dako
NKH1	—	Natural killer cell	Coulter
Leu 11b	CD16	Natural killer cell Neutrophil	Becton-Dickinson
Myeloid and monocyte markers			
MY4	CD14	Myelomonocytic	Coulter
MY7	CD13	Myelomonocytic	Coulter
MY9	CD33	Myelomonocytic	Coulter
MO2	CD14	Myelomonocytic	Coulter
OKM1		Myelomonocytic suppressor T cell, natural killer cell	Ortho (Raritan, NJ)
LeuM1	CD15	Myelomonocytic	Becton-Dickinson
Muramidase	—	Myelomonocytic	Dako
Alpha-1 antitrypsin	—	Myelomonocytic	Dako
Alpha-1-anti-chymotrypsin	—	Monocytic	Dako

investigate the clonality and cell lineage of various lymphoid malignancies [1–3]. The presence of a new rearranged band(s) on Southern analysis using the appropriate DNA probe provides a reliable marker of monoclonality [4–6]. The polymerase chain reaction (PCR), which is a quicker and more sensitive method, can also be used to detect the rearrangement [7–9]. A straightforward one stage PCR gives a sensitivity of 0.1% and this can be improved to 0.02% by performing a two stage nested or semi-nested reaction [8].

The T-cell receptor delta gene (TCRδ) is found to be clonally rearranged in many cases of T-cell malignancies [2,4,10]. In this study, the TCRδ gene rearrangement was used as the marker of MRD. A relatively simple but specific and sensitive method of detection of MRD in these patients was investigated. The rearrangement was first analysed by direct sequencing of the PCR product and a clonal specific primer was constructed for each individual case to perform a second PCR.

MATERIALS AND METHODS

Patients and Clinical Materials

Forty patients with acute leukaemia and lymphoma of T-cell lineage were studied. There were 18 non-Hodgkin's lymphoma (NHL) and 22 acute lymphoblastic leukaemia. The diagnosis was based on peripheral blood, bone marrow, and/or lymph node biopsy examination. The cell lineage was confirmed by immunophenotyping using a panel of monoclonal antibodies (Table I). Peripheral blood samples from 10 normal individuals were used as negative control. Specimens were collected prior to chemotherapy and malignant cell constituted at least 10% of cell nucleated cells in the specimens.

Southern Blot Analysis

High-molecular-weight DNA was extracted from the peripheral blood and bone marrow samples containing malignant cells. Ten micrograms of each DNA sample was digested independently with three restriction enzymes: EcoRI, BamHI, or HindIII, under conditions recommended by the manufacturer (New England Biolab, Beverly, MA). Digested products were electrophoresed overnight in a 0.7% agarose gel and transferred to nylon filter by Southern blotting. Filters were hybridised to the ^{32}P nick-translated radiolabelled TCRδ gene joining region probe (JδS16 probe, a 1.5 kb SacI Jδ1 fragment kindly provided by Drs. T. Boehm and T.H. Rabbitts, Cambridge, United Kingdom) [11]. After washing under stringent conditions (2 × 55°C and 1 × 60°C in 0.1 × SSC and 0.1% SDS washing buffer), autoradiography was carried out for 24–72 hours at −70°C.

Polymerase Chain Reaction Amplification (TCRδ PCR)

A pair of primers were designed to amplify the V-D-J recombination of the TCRδ gene in leukaemia and lymphoma of T-lineage [12]. Figure 1 shows the sequences and positions of the primers. The reaction mixture for PCR (50 μL) contained 0.5 μg template DNA, 50 pM primers, 1.25 mM dNTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 1 U Taq polymerase (Cetus, Norwalk, CT). Following heating to 94°C for 5 min, 35 cycles of denaturation (1 min at 94°C), annealing (1 min 30 sec at 56°C), and extension (1 min 30 sec at 72°C) were performed. Amplified DNA was electrophoresed on an 8% polyacrylamide gel (PAG) in 1 × TBE buffer, DNA was stained using ethidium bromide and visualised under ultraviolet light.

Direct Sequencing of PCR Products

The PCR products of four positive cases of T-acute lymphoblastic leukemia (T-ALL) were sequenced. Aliquots of 50 μL of each reaction product were separated on an 8% PAG. After ethidium bromide staining, the rearranged bands were visualised under ultraviolet light and were excised from the gel. They were eluted in two volumes of elution buffer pH 8.0 (0.5 mM ammonium acetate, 1 mM EDTA) in 37°C water bath overnight [13]. The buffer containing the DNA product was precipitated in 2 volumes 95% ethanol and 0.1 volume 2M NaAc, pH 5.6, dried and dissolved in 20 μl dH_2O. One μg each of the sequencing primer, VDS (Fig. 1) and the DNA product were included in the sequencing reaction using Sequenase Version 2.0 (United States Biochemical, Cleveland, OH) under the manufacturer's instructions [12]. Using ^{35}S labeling, it was run in denaturing 8% polyacrylamide sequencing gel. The gel was dried and autoradiography was performed for 1–2 days at room temperature.

Clonal Specific Polymerase Chain Reaction Amplification (CS-PCR)

From the sequences of the V-D-J region, a 3′ anti-sense primer was designed and synthesized for each of the 4 cases of T-ALL studied. Clonal specific PCR was performed using a 5′ sense primer (T2, Fig. 1) and the clonal specific 3′ anti-sense primer [14]. Seventy nanograms each of the primers and 0.5 μg template DNA with other standard reagents were used in a 50 μL reaction mixture. The reaction was started at 94°C for 5 min and followed by denaturation (94°C for 1 min), annealing (68°C for 1 min), and extension (72°C for 1 min) for 35 cycles. The product was stained in ethidium bromide and visualised under ultraviolet light. The sensitivity of the technique was determined by serial dilution of DNA specimen of known quantity of leukemic cells from patients with DNA obtained from peripheral blood of normal individuals.

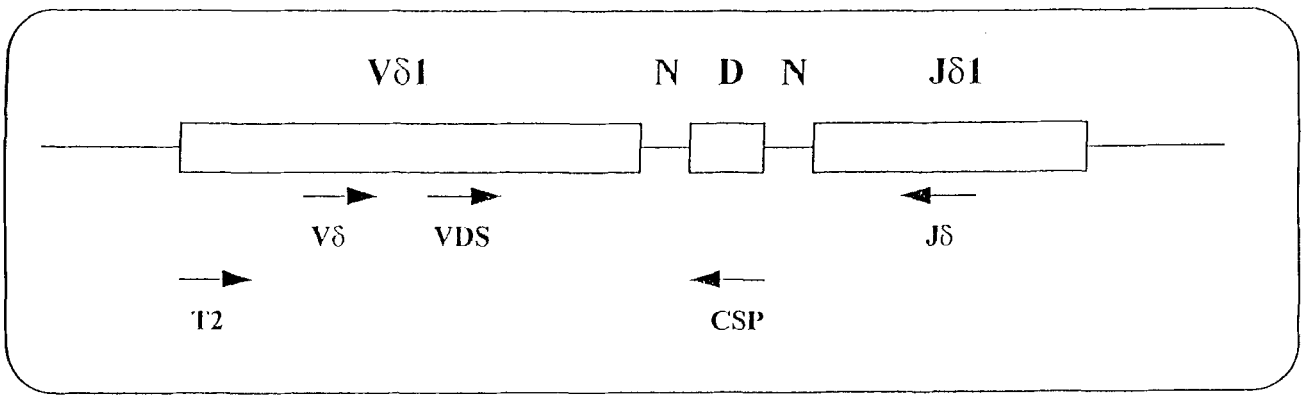
RESULTS

Southern Blot Analysis

DNA specimens containing malignant leukemic or lymphoma cells obtained from patients' peripheral blood, bone marrow, or lymph node were examined for TCRδ gene rearrangement by Southern analysis using the JδS16 probe. Seven of the 22 T-ALL (32%) and 5 of 18 (28%) T-cell lymphoma showed clonal rearrangement. All specimens from the normal control samples had germline pattern.

Polymerase Chain Reaction Amplification (TCRδ PCR)

Both Southern positive and negative samples were studied by PCR amplification. Six of the 7 (86%) Southern positive T-ALL and 4 of the 5 (80%) Southern positive T-cell lymphoma were TCRδ PCR positive. Clonal rearrangement of the TCRδ gene was indicated by the presence of one or two discrete DNA fragments of sizes between 180 and 220 bp (Fig. 2). All Southern negative cases as well as the blank and normal controls were TCRδ PCR negative except a case of Southern negative T-cell lymphoma (Fig. 2: lane 10, #302) which showed three discrete bands probably indicating oligoclonality. The intensity of PCR products for lanes 8 and 10 was greatly diminished compared to the other PCR-positive lanes, which indicated that probably relatively smaller proportion of the cells were involved. Also, the absence of a different level of signal among the bands in lane 10 indicated that the various clones were equally represented in the malignant population. The normal control showed a smear which represented normal polyclonal



Primers for TCRδ PCR

5' Sense (Vδ): 5' - AAAGTGGTCGCTATTCTGTC - 3'
3' Anti-sense (Jδ): 3' - TGGTTCCACAGTCACACGGG - 3'

Primers for DNA Sequencing of the V-D-J Region

VDS: 5' - AAAGCAGCGAAATCCGTCGCCTTA - 3'

Primers for CS-PCR
5' Sense Primer (T2): 5' - ACTCAAGCCCAGTCATCAGT - 3'
3' Anti-sense Primers (CSP): #181 5' - GTGTTCCCGGCCCTTATCCC - 3'
#448 5' - CCCAGCCCTTGTAGAGAGGCC-3'
#654 5' - AAGGAGACCGGTCCCAGGTTAA - 3'
#999 5' - ACTGTCGGGGGCTTAACGGGG - 3'

Fig. 1. A schematic diagram showing the relative positions and DNA sequences of the primers used for TCRδ PCR, DNA sequencing of the V-D-J regions and CS-PCR. CSP = 3′ anti-sense clonal specific primers.

rearrangement. The sensitivity was determined by serial dilution of a known quantity of DNA specimens of leukemic cells with DNA from normal control. This technique detected 0.1% malignant cells.

Direct Sequencing of PCR Products

The PCR products of four cases of T-ALL (#181, 448, 654, and 999) showing clonal pattern by TCRδ PCR amplification were successfully sequenced. Their DNA sequences are shown in Figure 3 together with the germline sequence. The rearrangement often involved parts of the Dδ1, Dδ2, and Dδ3 regions with additional nucleotides added to the N and P regions between them. The mean number of nucleotides in the N-D-N regions was 27.75 and the mean proportion of N and P nucleotides was 59.5%. From these data, 3′ anti-sense primers of 20–23 bp in sizes were designed and synthesized for each of the four cases as shown in Figures 1 and 3.

Clonal Specific Polymerase Chain Reaction Amplification (CS-PCR)

Clonal specific PCR amplification was performed using the T2 5′ primer and either one of the clonal specific 3′ anti-sense primers, producing a discrete band of 280–320 bp in size in the positive cases. Specificity of the technique was determined by amplifying 6 TCRδ PCR positive, 10 TCRδ PCR negative, and 10 normal control specimens. They were all negative except with the corresponding cases. The sensitivity was determined by serial dilution of known quantity of DNA specimens of leukemic cells with DNA from normal control. Figure 4 shows that the technique detected with confidence the rearranged band in a mixture containing 0.003% of malignant cells.

Study of Follow-Up Marrow Samples

Marrow specimens obtained at diagnosis and subsequent follow-ups from the 4 T-ALL patients were exam-

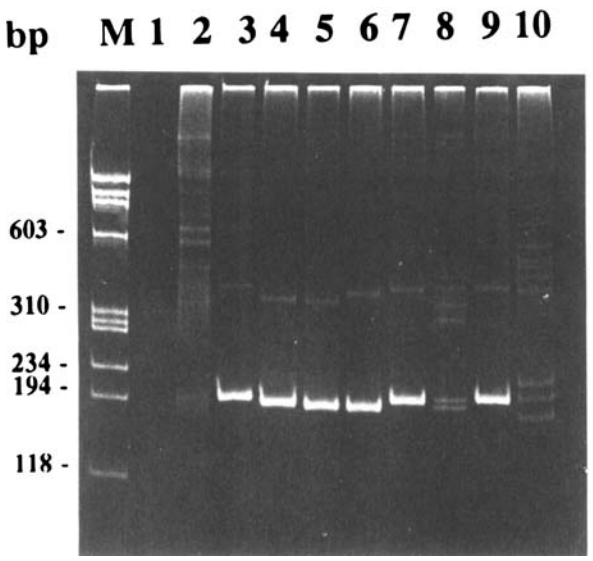


Fig. 2. DNA specimens of six patients with T-ALL and two with T-cell lymphoma. The TCRδ gene rearrangement was detected by PCR amplification of the V-D-J region. Amplified DNA was electrophoresed in 8% polyacrylamide gel and visualised by ethidium bromide and ultraviolet light. Lane 1 = blank control; lane 2 = normal control; lanes 3–8 = T-ALL (#181, #448, #654, #999, #937, and #533); lanes 9, 10 = T-cell lymphoma (#412 and #302).

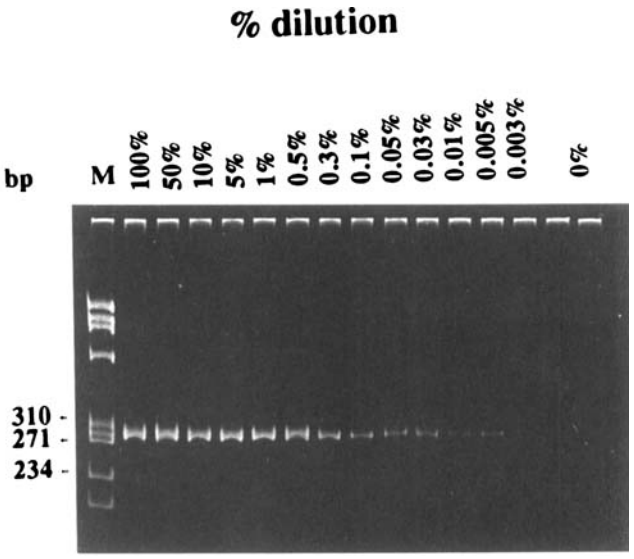


Fig. 4. DNA extracted from the marrow of a T-ALL patient #654 (marrow blast count = 95%) was serially diluted with that of a normal control. CS-PCR was performed. Under the ultraviolet light and on the original Polaroid film, it was possible to visualize the presence of the rearranged band at the level of 0.003% dilution.

ined morphologically and studied by Southern analysis, TCRδ PCR, and CS-PCR. The results are summarised in Figure 5. The first patient (#654) had a CR following induction chemotherapy and is alive in continuous CR by morphology for more than 3 years from diagnosis. All follow-up marrow samples were negative by Southern analysis, TCRδ PCR, and CS-PCR. The second patient (#999) also had a CR following induction chemotherapy but the disease relapsed at 18 months by morphology. The initial follow-up marrow specimens were negative by Southern analysis and TCRδ PCR but were positive by CS-PCR. Unfortunately, DNA samples at relapse were not available for analysis. The third and fourth patients (#448 and 181) both had not achieved a CR and died of progressive disease. All their follow-up marrow samples were positive by morphology, Southern analysis, TCRδ PCR, and CS-PCR.

DISCUSSION

Clonal TCRδ gene rearrangement has been found in many cases of T-cell malignancies and has been successfully used as a marker for minimal residual disease [5,7]. As the TCRδ gene has a very limited germline configuration and its Vδ1 and Jδ1 regions are preferentially used in complete recombination [15–17], PCR can be conveniently used for detection of clonal rearrangement. Moreover, an extraordinary diversity is found at the rearranged

	Vδ1	N	Dδ1	N	Dδ2	N	Dδ3	N	Jδ1
Germline	ACTTTTGTGCTCTTGGGGAACT		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAAACTCAT
Patient									
#181	ACTTTTGTGCTCTTGGGGAACT	aGG	TAGT		CCT		ACTGGGGGATA	AGGGCCGGGA	ACACCGATAAACTCAT
#448	ACTTTTGTGCTCTTGGGG	CCT			CTAC	AAGGg	CTGGGGGAT	GCC	ACACCGATAAACTCAT
#654	ACTTTTGTGCT	TTC	TA	C	CCT	GGTTAACCT	GGGA	CCGGTCTCCTC	ACACCGATAAACTCAT
#999	ACTTTTGTGCTCTTGGGGA				TCC	CCCGTTAAGCCCCC	GA	CAg	ACACCGATAAACTCAT

Fig. 3. The DNA sequences of the germline and rearranged V-D-J regions of four cases of T-ALL. Nucleotides of the P regions are shown in small characters and the sequences used as the 3′ anti-sense clonal specific primers are underlined.

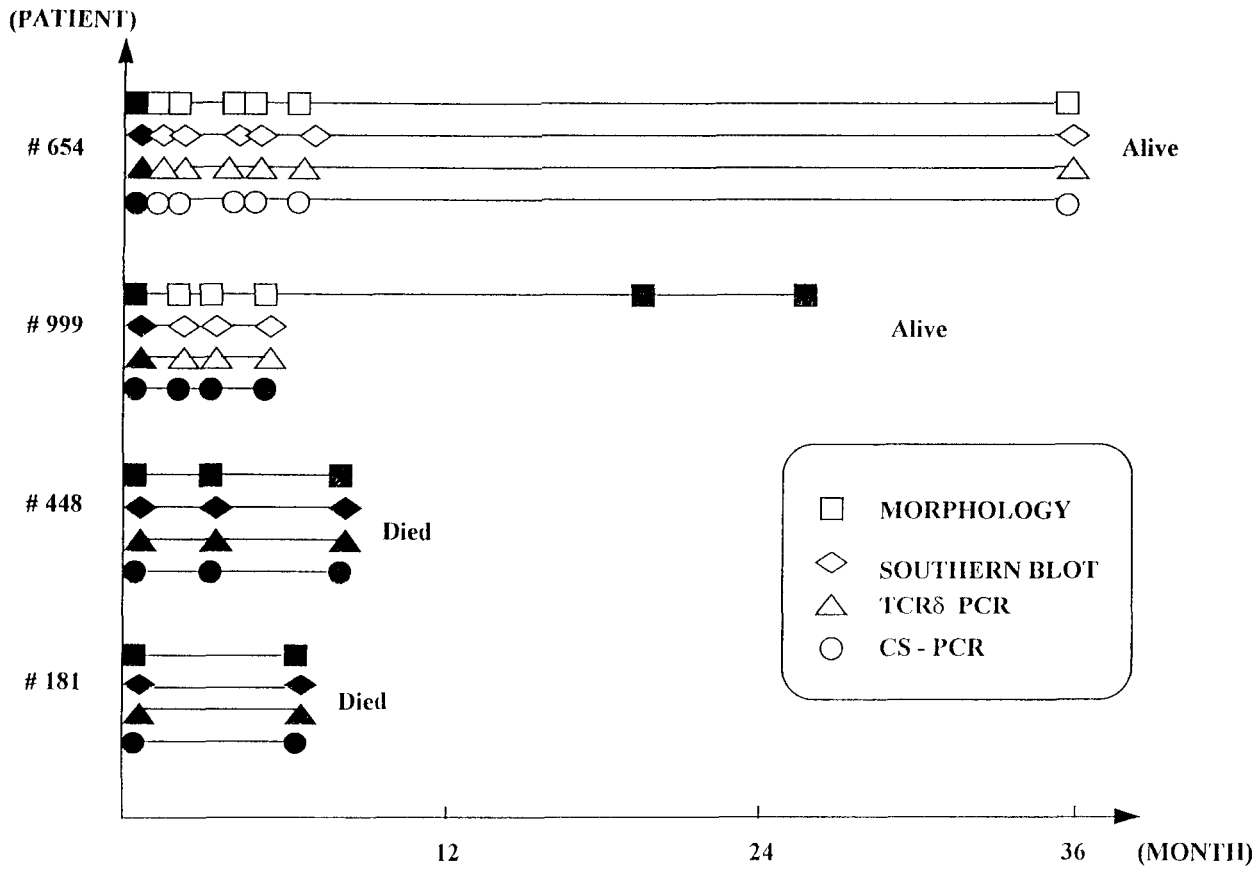


Fig. 5. The results of morphological examination, Southern analysis, TCRδ PCR, and CS-PCR performed using the marrow specimens obtained at diagnosis and subsequent follow-ups for four patients with T-ALL. Solid markers indicate positive results.

V-D-J region due to the recombination of the D regions and insertions/deletions at the N and P regions [18,19]. This permits the design and synthesis of clonal specific oligonucleotides for use as primer or probe for detection of MRD [14,20].

Clonal TCRδ rearrangement has been found to occur commonly in T-ALL in the Western population and around 50 to 60% of their T-ALL were positive by Southern analysis [21,22]. A lower incidence of 32% was observed in our T-ALL cases. This may reflect the heterogeneity of patients. An even low incidence of 28% was also seen in our cases of T-cell lymphoma, of which the majority were of the peripheral T-cell type. The finding did not correlate with the tissue source. Also, there was no correlation with the relative percentage of malignant cells in the specimen.

The TCRδ rearrangement can be successfully detected by TCRδ PCR in most of the Southern positive specimens. False positives were not seen in the normal controls. A pattern of oligoclonality was observed in one Southern negative case of T-cell lymphoma. On the other hand, false negativity was found in 14% of the Southern positive T-ALL and 20% of the T-cell lymphoma. Despite the small number of cases studied, these findings were similar to that of the other reported series [6,12,22]. False negativity can be explained by the biased preferential rearrangement of the other V or J segments or their inversion during recombination.

Looking at DNA sequences, it appeared that the rearranged V-D-J region was specific to individual cases and had enough diversity to allow us to design and synthesize clonal specific primer for each case. This improved the specificity and provided a higher sensitivity of down to 0.003% compared with TCRδ PCR in detecting MRD. The N and P regions occupied almost 60% of the rearranged V-D-J region. As the N regions were rich in nucleotides G and C, unlike the D regions, clonal specific 3′ anti-sense primers were constructed according to the DNA sequences of mainly the N regions. This avoided the amplification of the germline sequence and hence further enhanced the specificity of the technique. T2 was chosen to be the 5′ primer for all cases [14]. This produced a relatively long PCR product of around 330 bp in length giving a dense band under ethidium bromide and ultraviolet light visualisation.

Without using radiolabelling, our technique is simpler

but has a sensitivity comparable to that of the method of using radiolabelled clonal specific probe for hybridization. The technique was applied to study the follow-up marrow samples of the 4 T-ALL patients. Patient #654 was in morphological CR for more than 3 years and had persistently negative Southern, TCRδ PCR, and CS-PCR results. Follow-up marrow samples from patient #999 initially had persistently positive CS-PCR results although they were morphologically and TCRδ PCR negative, indicating persistent presence of leukaemic cells in the marrow in the range of 0.003 to 0.01% following induction chemotherapy. The patient had a frank leukaemic relapse at 18 months. This CS-PCR technique appeared to predict the relapse of leukemia. The other two patients (#448 and 181) had persistent disease even by conventional morphological examination and both TCRδ PCR and CS-PCR studies were positive as expected.

CS-PCR is a highly specific and sensitive technique in detecting MRD. It can potentially be used to detect occult disease in morphologically normal peripheral blood marrow and other tissues of patients with leukemia and lymphoma at diagnosis. Also, studies on serial follow-up samples may detect residual leukemia or recurrent disease in leukemic patients in apparent complete remission by morphological criteria. Furthermore, it provides a sensitive way to define the necessity or effectiveness of in vitro purging of the marrow harvested from patients for autologous marrow transplantation. All these potential applications require further clinical evaluation and correlation.

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